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Temporal Regulation of Hyaluronan and Proteoglycan Metabolism by Human Bone Cells *in Vitro**

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Osteoblasts elaborate a dynamic extracellular matrix that is constructed and mineralized as bone is formed. This matrix is primarily composed of collagen, along with noncollagenous proteins which include glycoproteins and proteoglycans. After various times in culture, human bone cells were labeled with [^{35}S]sulfate, [^{3}H] leucine/proline, or [^{3}H]glucosamine and the metabolism of hyaluronan and four distinct species of proteoglycans (PGs) was assayed in the medium, cell layer, and intracellular pools. These cells produce hyaluronan (M_r ~ 1,400,000; a chondroitin sulfate PG (CSPG), M_r ~ 600,000; a heparan sulfate PG (HSPG), M_r ~ 400,000; and two dermatan sulfate PGs with M_r ~ 270,000 (biglycan, PG I) and M_r ~ 135,000 (decorin, PG II) that distribute between the medium and cell layer. Two days following subculture, 12 h [^{35}S]sulfate steady-state labeling yielded a composition of 24, 27, 31, and 18% for total CSPG, HSPG, biglycan, and decorin, respectively. While HSPG and decorin levels and distribution between medium and cell layer remained relatively constant during steady-state labeling at different times in culture, CSPG and biglycan levels increased dramatically at late stages of growth, and their distribution changed throughout culture. These results were independent of cell density, media depletion, and labeling pool effects. In contrast, hyaluronan synthesis was uncoupled from PG synthesis and apparently density-dependent. Pulse chase labeling at different stages of culture showed that the CSPG and decorin behaved as secretory PGs. Both HSPG and biglycan underwent catabolism, with HSPG possessing a $t_{1/2}$ of 8 h and biglycan a $t_{1/2}$ of 4 h. While the rate of HSPG turnover did not appreciably change between early and late culture, that of biglycan decreased. The mRNA for decorin was constant, while that of biglycan changed during culture. These results suggest that each PG possesses a distinct pattern of cellular and temporal distribution that may reflect specific stages in matrix formation and maturation.

Proteoglycans (PGs)[1] are a ubiquitous family of biomolecules that consist of a core protein and one or more covalently attached sulfated glycosaminoglycan chains. Synthesis and sulfation of glycosaminoglycans occurs on genetically distinct acceptor core proteins within the Golgi, followed by rapid translocation to the cell surface (Roden, 1980). In contrast, the biosynthetic machinery for hyaluronan, which is an unsulfated glycosaminoglycan, is located at the plasma membrane and does not involve a covalently attached core protein (Philipson and Schwartz, 1984). PGs have been found associated with intracellular compartments (Pacifici *et al.*, 1983; Stow *et al.*, 1985a; Stevens 1987; Ripellino *et al.*, 1989), the cell surface (Kjellen *et al.*, 1981; Norling *et al.*, 1981; Hedman *et al.*, 1983; Rapraeger and Bernfield, 1983), extracellular matrices (Hascall and Hascall, 1981; Johansson *et al.*, 1985; Heremans *et al.*, 1988), and basement membranes (Stow *et al.*, 1985b). Although no well defined functional role has yet been elaborated for PGs, they have been implicated in cell adhesion to substratum (Laterra *et al.*, 1983; Wightman *et al.*, 1986), maintainence of connective tissue resilience (Hardingham and Muir, 1972; Kempsen, 1973; Handley *et al.*, 1985), cell surface and cytoskeletal interactions (Rapraeger and Bernfield, 1982; Woods *et al.*, 1984, 1985; Carey and Todd, 1986), fibrillar collagen interactions (Scott and Oxford, 1981; Scott, 1988; Scott and Haigh, 1988; Vogel *et al.*, 1987; Uldberg and Danielson, 1988), acting as receptors for thrombospondin (Kaesberg *et al.*, 1989) and transforming growth factor β (Cheifetz *et al.*, 1988), and in sequestering basic fibroblast growth factor (Saksela *et al.*, 1988), granulocyte-macrophage colony stimulating factor (Gordon *et al.*, 1987), and interleukin 1 (Roberts *et al.*, 1988). Any understanding of the physiological function(s) of PGs must involve definition of both the localization and metabolism of PGs.

Although functional significance is ultimately addressed *in vivo*, in the case of human cellular metabolism an *in vitro* model system facilitates such studies. The numerous sites for potential regulation of PG levels and distribution between the various cellular compartments make the interpretation of *in vitro* changes in PG metabolism difficult. Investigations of PG biosynthesis involving *in vitro* systems and radiolabeled precursors typically do not follow more than one PG species, nor do they take into account possible alterations in the cell's metabolic potential as a result of *in vitro* variables such as cell cycle, density, or cultural age.

Bone cells in culture produce hyaluronan and four distinct species of medium and pericellular matrix-associated PGs (Table I and Hunter *et al.*, 1983; Beresford *et al.*, 1987; Ecarot-Charrier and Broekhuyse, 1987). The four PGs, a large chondroitin sulfate PG (CSPG), a heparan sulfate PG (HSPG), two smaller dermatan sulfate PGs (decorin and biglycan; Fisher *et al.*, 1989), and hyaluronan, comprise a complex system well suited for studying PGs and factors that regulate their metabolism and interactions as an extracellular matrix is secreted, deposited, and matured *in vitro*. In modeling *in vivo* events (matrix deposition and bone formation) with such

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[1] The abbreviations used are: PG, proteoglycan; CSPG, chondroitin sulfate proteoglycan; HSPG, heparan sulfate proteoglycan; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TABLE I

Hyaluronan and proteoglycans in human bone cell cultures

Species[a]	Name[b]	Intact PG	M_r[c]	
			Core protein	*Glycosaminoglycan*
Hyaluronan	Hyaluronan			1,400,000[d]
Chondroitin sulfate	CSPG	600,000	390,000 & 340,000	40,000
Heparan sulfate	HSPG	400,000	82,000–83,000[e]	55,000–65,000
Dermatan sulfate	Biglycan	270,000	52,000 & 49,000	42,000–45,000
Dermatan sulfate	Decorin	135,000	52,000	42,000–45,000

[a] Identity of glycosaminoglycan component as determined by enzyme susceptibility and/or resistance.
[b] The name biglycan has been applied to the larger of a class of two small interstitial proteoglycans (Fisher *et al.*, 1989), and decorin has been proposed for the smaller proteoglycan (Ruoslahti, 1988).
[c] The molecular weights for the proteoglycans and their components were calculated from SDS-PAGE analysis of [^{3}H]leucine/proline and [^{35}S]sulfate radiolabeled proteoglycans and components (Beresford *et al.*, 1987).
[d] The molecular weight of hyaluronan was determined using a 50 × 1.0-cm TSK-GEL HW 75(F) column equilibrated in 6 M guanidinium HCl, 0.1 M KH_2PO_4, pH 6.0 and 0.5% Triton X-100, eluted at a flow rate of 0.5 ml/min, and calibrated with protein standards (Fedarko *et al.*, 1990).
[e] The molecular weight of the reduced HSPG core protein generated from heparitinase digestion was determined using a Superose 6 column equilibrated in 6 M guanidinium HCl, 0.1 M KH_2PO_4, pH 6.0, eluted at a flow rate of 0.4 ml/min and calibrated with protein standards (Fedarko *et al.*, 1990).

an *in vitro* system (bone-derived cell culture), it is necessary to distinguish between biosynthetic patterns that reflect the bone cell phenotype and those that are imposed by the culture system. Thus, as a prerequisite to studies of PGs in diseased states of bone and in developmental studies, it is necessary to define the metabolic pattern of PGs in the *in vitro* system. In this report, we characterize *in vitro* PG and hyaluronan metabolism in order to: 1) separate events involved with matrix deposition from those correlated with cell density, serum effects, and changes in labeling pool activity (*in vitro* limitations); 2) kinetically distinguish pericellular from extracellular matrix components; and 3) define the compartmentalization and turnover of these macromolecules as matrix is formed and matured.

MATERIALS AND METHODS

Ultrapure guanidinium HCl, glycine, and Tris-HCl were obtained from Bethesda Research Laboratories. Formamide and barium nitrite were obtained from Fluka. TSK-GEL ToyoPearl HW 40(S), HW 65(S), HW 75(F), and HW 65(S) DEAE resins were obtained from Thomson Instrument Company. *Streptomyces* hyaluronidase, chondroitinase ABC (*Proteus vulgaris*), AC II (*Arthrobacter aurescens*), and heparitinase (heparin lyase II) were obtained from ICN Biochemicals. All other reagents were of best analytical grade available.

Cell Culture—Human trabecular bone was cultured as described previously (Gehron Robey and Termine, 1985). Briefly, trabecular bone of femoral head or patella origin was minced, treated with 250 units/ml collagenase (type IV, Sigma) in Dulbecco's modified essential medium (Biofluids), 100 units/ml penicillin-streptomycin (Biofluids), and 2 mM glutamine (Biofluids) without fetal bovine serum. The collagenase-treated bone chips were cultured in low calcium medium ($CaCl_2$-free Dulbecco's modified essential medium) containing 10% heat-inactivated fetal bovine serum, glutamine, penicillin-streptomycin, and 50 μg/ml ascorbate (Sigma).

Steady-state and Pulse Chase Proteoglycan Labeling—Human bone cells were passaged from primary culture just prior to confluence and seeded at a density of 10,000 cells/cm^2. The cultures were changed to a mineralization medium containing 1.8 mM calcium, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium (Collaborative Research), and 10 mM β-glycerol phosphate (Sigma). Cultures were fed each day and on days 1–5, 9, 12, 16, 19, and 22 duplicate dishes were taken for labeling and cell counts. The cells were steady state-labeled for 12 h with 50 μCi/ml [^{35}S]sulfate or 10 μCi/ml [6-^{3}H] glucosamine (Du Pont-New England Nuclear) and cell number was determined on trypsinized cells by the use of a Coulter Counter (Hialeah, FL). After labeling, the medium pool was generated by removing the labeling medium and rinsing the monolayer twice with phosphate-buffered saline (0.14 M NaCl, 0.005 M KCl, 0.0015 M KH_2PO_4, pH 7.4). The cell layer was extracted overnight at 4 °C in 0.05 M Tris buffer (pH 7.4) containing 4 M guanidinium HCl, 0.5 M EDTA, 0.005 M benzamidine, 0.01 M *N*-ethylmaleimide, 0.1 M 6-aminocaproic acid, 0.001 M phenylmethylsulfonyl fluoride, and 2% Triton X-100. The medium pools were diluted 1:1 with a 2 × stock of the extraction buffer. In some cases, an intracellular pool was analyzed by taking duplicate labeled cohort dishes whose cells had been released from the dish by 0.05% trypsin-EDTA treatment, pelleting the cells by centrifugation at 800 × *g* for 5 min, and dissolving the cell pellet in the Tris-guanidinium HCl buffer and extracting overnight at 4 °C.

For pulse-chase studies, cells were labeled with 100 μCi of [^{35}S] sulfate/ml and 10 μCi/ml [^{3}H]leucine and [^{3}H]proline for 0.5–5.5 h. After the 5.5-h pulse, the labeling medium was removed, the monolayer rinsed twice with Tris-buffered saline (pH 7.4), and then fresh medium lacking radioactive precursor but containing normal levels of cold sulfate, glutamine, proline, and leucine was added and the dishes returned to 37 °C. At 1, 3, and 5 h of the pulse and 2, 4, 6, 12, 24, and 48 h of the chase, dishes were harvested as in the continuous steady-state labeling experiments.

Quantitation of Proteoglycan Levels—PGs were isolated and quantified using an HPLC system recently developed (Fedarko *et al.*, 1990). Briefly, unincorporated radioactive precursor was removed by HPLC desalting utilizing a 1.0 × 25-cm ToyoPearl TSK-GEL HW 40(S) equilibrated in 50% formamide, 40 mM KH_2PO_4 (pH 6.0), and 0.5% Triton X-100. During this step the total amount of label incorporation into macromolecules was determined as the excluded polymer-labeled material by liquid scintillation counting of fraction aliquots.

The incorporation of label into hyaluronan was determined by chromatography of an aliquot of the [^{3}H]GlcN-labeled pool before and after digestion with hyaluronidase (3.3 turbidity-reducing units/ml in a 0.02 M acetate buffer (pH 5.0)) on a 1.0 × 25-cm ToyoPearl TSK-GEL HW 40(S) column equilibrated in the formamide buffer system.

Determination of [^{3}H]leucine/proline or [^{3}H]GlcN label incorporation into proteoglycans was done by two successive column isolation steps followed by SDS-PAGE analysis and liquid scintillation counting of dissolved gel slices. Radiolabeled material was desalted by the TSK-GEL HW 40(S) system, and the entire excluded pool was directly injected on a Nucleogen DEAE 4000-10 column (Machery Nagel). The gradient system consisted of an initial 20-min isocratic segment at 0.1 M potassium acetate, followed by a 55-min linear gradient to 0.4 M potassium acetate to resolve glycoproteins from hyaluronan, and then a 45-min linear gradient to 1.0 M potassium acetate to elute the proteoglycans. An aliquot of the proteoglycan pool was then analyzed by SDS-PAGE and label incorporation into proteoglycans determined by liquid scintillation counting of H_2O_2 disolved 1-mm gel slices (see below).

The distribution of the [^{35}S]sulfate label among the PGs was determined by two methods. The first method involved liquid scintillation counting of solublized 1-mm segments from 3 to 15% SDS-polyacrylamide gels (see below). Similar values were obtained by the second method where the PGs were resolved by analytical anion exchange chromatography using a Nucleogen DEAE 4000-7 column (Machery Nagel) developed with a potassium acetate gradient. Proteoglycans were identified by enzyme susceptibility or resistance. Heparitinase (20 milliunits/ml), chondroitinase ABC (40 milliunits/

ml), and chondroitinase AC II (40 milliunits/ml) digestions were carried out as described previously (Beresford *et al.*, 1987).

Polyacrylamide Gel Electrophoresis—Separation of PGs by SDS-PAGE was carried out as described previously (Beresford *et al.*, 1987). The gradient system consisted of 3–15% acrylamide and 0.06–0.08% bisacrylamide. Samples were reduced with 2 mM dithiothreitol and heated at 100 °C for 10 min prior to loading. Electrophoresis was carried out at a constant current of 9 mA/gel for 14 h or until the prestained carbonic anhydrase molecular weight marker (30,000) reached the bottom of the gel. Gels were processed for fluorography using dimethyl sulfoxide and 2,5-diphenyl-1,3,4-oxadiazole as described previously. For liquid scintillation counting, gels were cut into 1-mm segments using a Mickel gel-cutting apparatus (Brinkman Instrument Co.) and solubilized by H_2O_2 treatment (Beresford *et al.*, 1987).

Northern Analysis of Proteoglycan mRNA—The levels of mRNA for biglycan and decorin were assayed at various times in culture by the method of Huarte *et al.* (1987). Briefly, cells were chilled to 4 °C, disrupted with 4 M guanidine thiocyanate, supplemented with 20 μg of *Escherichia coli* tRNA, and then chloroform/phenol-extracted twice. The RNA was separated on 1.7% agarose-formaldehyde gels by electrophoresis at 10 mV overnight. ^{32}P-Labeled probes for biglycan, decorin (PG I and PG II), and glyceraldehyde-3-phosphate dehydrogenase (Piechacyzk *et al.*, 1984) were labeled as described (Fisher *et al.*, 1989). After hybridization, [^{32}P]mRNA bands were visualized by autoradiography, cut from the nitrocellulose paper, and thoroughly dried before dissolving each sample in a toluene scintillation fluid containing 6 g/liter 2,5-diphenyl-1,3,4-oxadiazole/2,2′-*p*-phenylenebis-[5-phenyloxazole].

RESULTS

Steady State Labeling and Proteoglycan Distribution—Human bone cells passaged from primary culture prior to confluence, seeded at a density of 20,000 cells/cm^2 and maintained for 48 h in mineralization medium, were labeled with [^{35}S] sulfate, [^{3}H]leucine/proline, or [^{3}H]GlcN for 12 h. Following removal of unincorporated [^{3}H]GlcN or [^{3}H]leucine/proline radioactive precursor, the incorporation of ^{3}H-label into proteoglycans was determined by separating proteins/glycoproteins, hyaluronan, and PGs by anion exchange chromatography on Nucleogen 4000-10 DEAE column (Fig. 1*a*). Radiolabeled macromolecules were identified by rechromatography on the TSK-GEL HW 40(S) gel filtration column before and after enzyme digestions (*Streptomyces* hyaluronidase, chondroitinase ABC, or heparitinase). Under the conditions developed for the anion exchange elution, with [^{3}H]GlcN labeling, the excluded material, peak 1, was found to consist of 90% [^{3}H]GlcN glycoproteins and 10% residual unincorporated label. A series of overlapping peaks (*peak 2*, Fig. 1*a*) eluted next at 0.1–0.25 M potassium acetate and were also found to be glycoproteins. Peak 3, which eluted at 0.4 M potassium acetate corresponded to [^{3}H]hyaluronan, and peak 4, eluting at high salt, contained the [^{3}H]PGs. An aliquot of the [^{3}H] PGs was then analyzed by SDS-PAGE and the amount of label present in each PG determined by liquid scintillation counting of dissolved 1-mm gel slices.

To define an initial *in vitro* "reference" state, the distribution of the [^{35}S]sulfate label among the PGs 48 h following subculture was determined by liquid scintillation of solublized 1-mm segments from 15-cm 3–15% polyacrylamide gels (Fig. 1*b*). The PGs on SDS-PAGE were resolved by size in the order of a CSPG (M_r ~ 600,000); HSPG (M_r ~ 400,000); biglycan (M_r ~ 270,000); and decorin (M_r ~ 135,000). The percent of total (medium plus cell layer) [^{35}S]PG pool had a composition of 24, 27, 31, and 18% for CSPG, HSPG, biglycan, and decorin, respectively. Similar values were obtained when the PGs were resolved by analytical anion exchange chromatography using a Nucleogen DEAE 4000-7 column developed with a potassium acetate gradient (Fig. 1*c*). When resolved by charge density, the medium [^{35}S]sulfate-labeled material

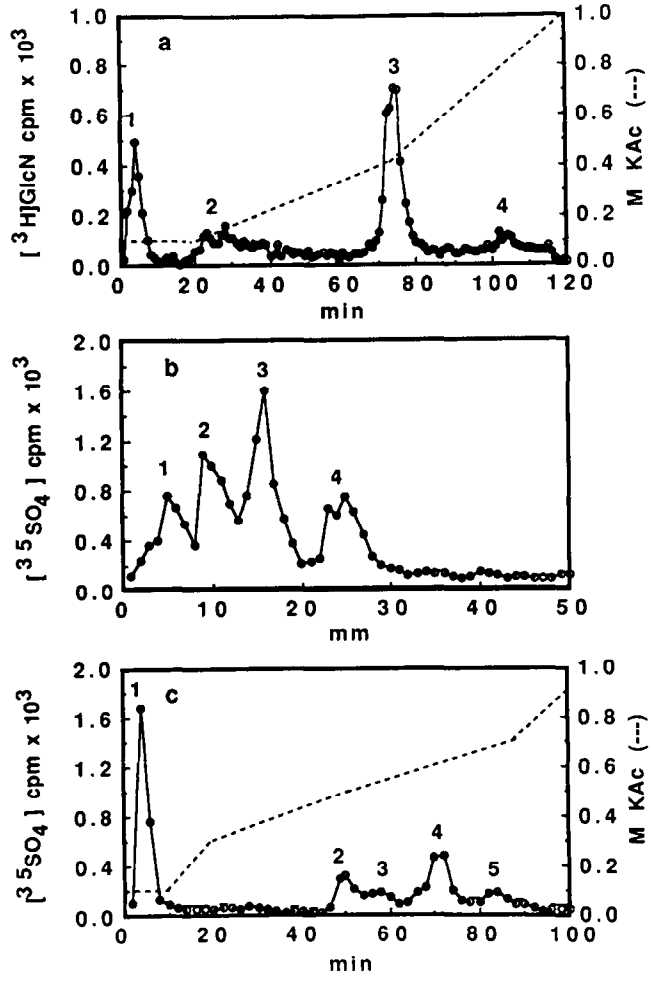

FIG. 1. **Quantitation of radiolabel into proteoglycans and hyaluronan.** Human bone cells were radiolabeled with either 10 μCi/ml [^{3}H]GlcN or 50 μCi/ml [^{35}S]sulfate for 12 h. [^{3}H]GlcN- or [^{35}S]sulfate-labeled material (*a*) was fractionated into glycoproteins (*peaks 1* and *2*), hyaluronan (*peak 3*), and proteoglycans (*peak 4*) by anion exchange chromatography using a Nucleogen 4000-10 DEAE column and a potassium acetate gradient. Incorporation of [^{35}S] sulfate into proteoglycans (*b* and *c*) was determined by either liquid scintillation counting of 1-mm slices of 3–15% acrylamide gels (*b*) or by analytical anion exchange using a Nucleogen 4000-7 DEAE column and a potassium acetate gradient (*c*). The [^{35}S]proteoglycans were resolved by SDS-PAGE (*b*) into four broad peaks that were identified as CSPG (*peak 1*), HSPG (*peak 2*), biglycan (*peak 3*), and decorin (*peak 4*). When [^{35}S]proteoglycans were separated by charge density on anion exchange (*c*), the proteoglycans eluted in the order of HSPG (*peak 1*), decorin (*peak 2*), biglycan (*peak 3*), and CSPG (*peak 4*).

eluted as five peaks. Residual unincorporated label and sulfated glycoproteins were eluted as a single unbound peak (peak 1, with 4% of peak 1 being sulfated glycoproteins and the rest unincorporated free $^{35}SO_4^{2-}$). The PGs eluted in the order HSPG (peak 2), decorin (peak 3), biglycan (peak 4), and CSPG (peak 5). PGs accounted for 80% of the [^{35}S] sulfate-labeled material, but only 10% of the total (medium plus cell layer) [^{3}H]GlcN-labeled material.

With time in culture, bone cells reach confluency (day 9) and begin to multilayer. Initially, very little extracellular matrix surrounds the cells; it increases dramatically as a function of time.[2] To investigate bone cell metabolism during the process of *in vitro* matrix accumulation, the steady state pattern of [^{3}H]GlcN and [^{35}S]sulfate incorporation was fol-

[2] B. R. Heywood and P. Gehron Robey, unpublished results.

lowed as a function of length of time in culture (Fig. 2, *a* and *b*). For both [^{3}H]GlcN and [^{35}S]sulfate, the total amount of steady state label incorporation on a per cell basis initially increased as a function of days in culture, approximately doubling 48 h after passage. The total label incorporated continued to increase, reaching a maximum by day 4–5, after which the level of label incorporated remained relatively constant. The proportion of the total [^{3}H]GlcN-labeled material incorporated into the cell layer typically increased from 35 to 40% of the total between day 1 and day 22 (Fig. 2*a*), while that of [^{35}S]sulfate-labeled cell layer material increased from 30% on day 1 to 50% by day 19 (Fig. 2*b*). Because the cell layer is a mixture of intracellular, cell surface, and matrix-associated pools, the observed increases could arise from a generalized increase in all three components of the cell layer or a specific increase in a particular pool. When the intracellular [^{35}S]sulfate-labeled pool was analyzed, there was only a slight increase of the intracellular pool as a percentage of the cell layer [^{35}S]sulfate-labeled material throughout time in culture (Fig. 2*b*). The majority of the intracellular [^{35}S]sulfate-labeled material (85%) was characterized as free dermatan and heparan sulfate glycosaminoglycan chains as indicated by the absence of a shift in gel permeation chromatography mobility upon treatment with alkaline $NaBH_4$ (data not shown). With an intracellular pool (composed of apparent catabolic products) remaining relatively constant, the increased incorporation of radiolabel into the cell layer reflects a specific increase in cell surface and/or matrix associated sulfated molecules. Thus, the observed pattern of radiolabel incorporation is consistent with the synthesis and deposition of an extracellular matrix with length of time in culture.

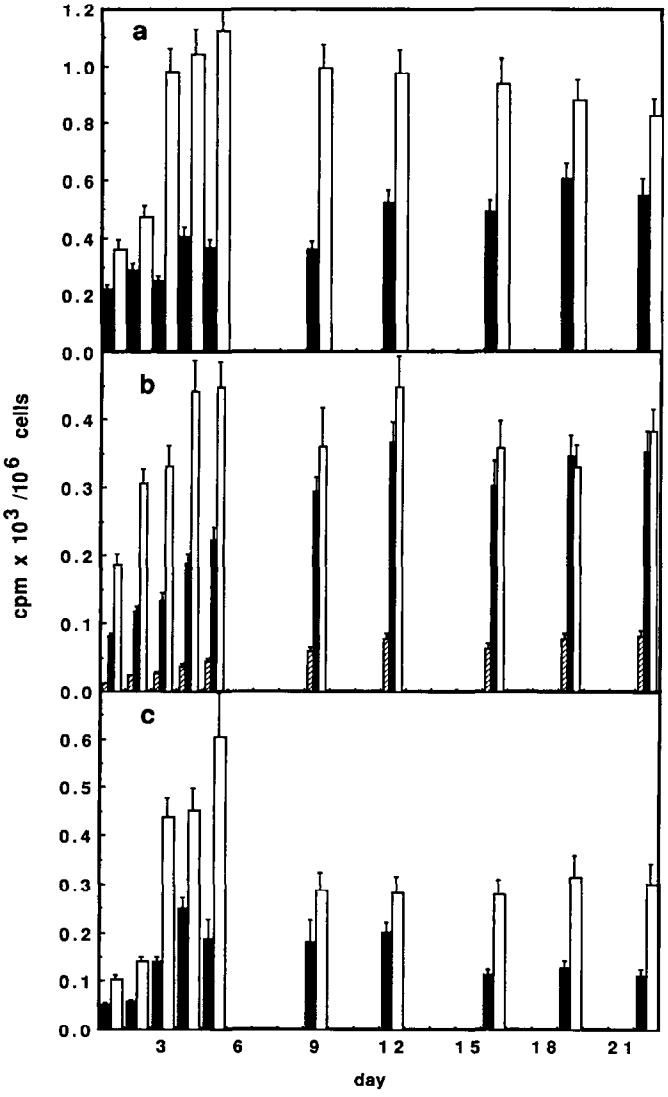

FIG. 2. **Steady-state labeling with [^{3}H]GlcN and [^{35}S]sulfate and days in culture.** Human bone cells seeded at 10,000 cells/cm^2 were radiolabeled with 10 μCi/ml [^{3}H]GlcN (*a*) or 50 μCi/ml [^{35}S] sulfate (*b*) for 12 h and the incorporation into medium (□) and cell layer (■) pools was determined. In the case of [^{35}S]sulfate-labeled material, the intracellular pool (▨) was also quantitated by trypsin-EDTA release of the cells from cohort dishes, pelleting the cells, and extracting the cell pellet (*b*). The incorporation of [^{3}H]GlcN into hyaluronan was determined by chromatography before and after hyaluronidase treatment (*c*).

To determine whether all cell surface and/or matrix associated components or only a specific few were increasing with length of time in culture, the radiolabel incorporation into hyaluronan and individual PGs was analyzed. When the incorporation of [^{3}H]GlcN into hyaluronan was quantitated, the majority of hyaluronan appeared in the medium and initially increased as a steady-state product until day 5. After day 5, the medium hyaluronan decreased to one-half its maximal level by day 9 and then remained constant while that in the cell layer decreased with time (typical results seen in Fig. 2*c*).

When steady-state levels of the individual [^{35}S]PGs were analyzed, large differences were seen with the progression of time in culture (Fig. 3). On day 1 in culture, CSPG, HSPG, biglycan, and decorin accounted for 17, 30, 28, and 25%, respectively, of the total PGs, although the levels in the medium were between two and three times that of the cell layer. By day 22, the distribution of [^{35}S]sulfate into total (medium plus cell layer) CSPG, HSPG, biglycan, and decorin was 11, 15, 48, and 15%, respectively and levels in the medium and cell layer and medium were approximately equal. An identical pattern of partitioning between the medium and cell layer was observed for [^{3}H]GlcN-labeled PGs (data not shown). On a per cell basis, the absolute amounts of the four PGs in the medium pool reached a maximum during days 3–5, whereas the steady-state-labeled PGs of the cell layer pool increased until they equaled or were greater than those in the medium pool by day 9. Both the HSPG and decorin showed only slight changes in either amount of label incorporated or in compartmentalization after 4 days in culture (Fig. 3, *b* and *d*). In contrast, the amount and distribution of the large CSPG and biglycan changed dramatically with length of time in culture (Fig. 3, *a* and *c*). The amount of CSPG in the cell layer increased linearly from day 1 to day 5, while cell layer biglycan increased linearly until day 12. Between day 2 and day 19, total biglycan increased almost 3-fold during 12 h steady-state labeling with the amount associated with the cell layer increasing 7-fold.

Effect of Cell Density on Proteoglycan and Hyaluronan Levels and Distribution—The observed partitioning between the medium and cell layer and the change in proportion between the different PG species could be attributable to differences in cell density at the various time points. To investigate the influence of initial cell density on PG and hyaluronan metabolism, bone cells were seeded at 10, 20, 40, and 60 × 10^3 cells/cm^2, allowed to recover for 24 h, labeled with [^{3}H]GlcN and [^{35}S]sulfate for 12 h, and the distribution of [^{3}H]GlcN- and [^{35}S]sulfate-labeled species was determined. When compared to the observed shifting distribution between the cell layer and the medium in long term steady state labeling with increasing time in culture, there was little if any change in distribution of total [^{35}S]PGs at the different densities shortly

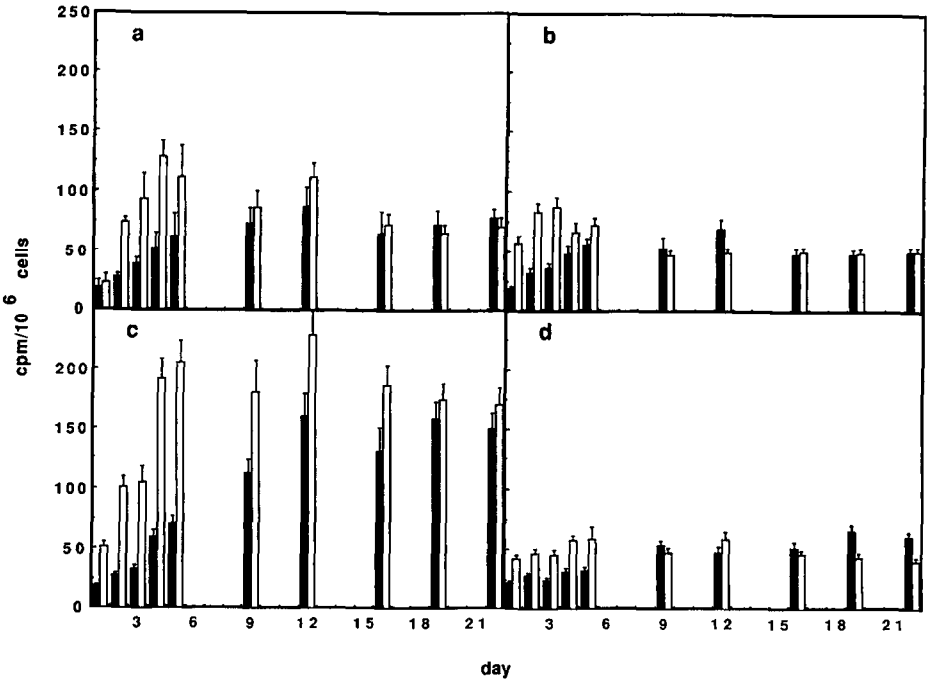

FIG. 3. **[^{35}S]Sulfate steady-state labeling of proteoglycans with days in culture.** Human bone cells were grown and labeled as in Fig. 2. The incorporation of [^{35}S]sulfate into the large CSPG (*a*), HSPG (*b*), biglycan (*c*), and decorin (*d*) medium (□) and cell layer (■) pools was determined as in Fig. 1*b*.

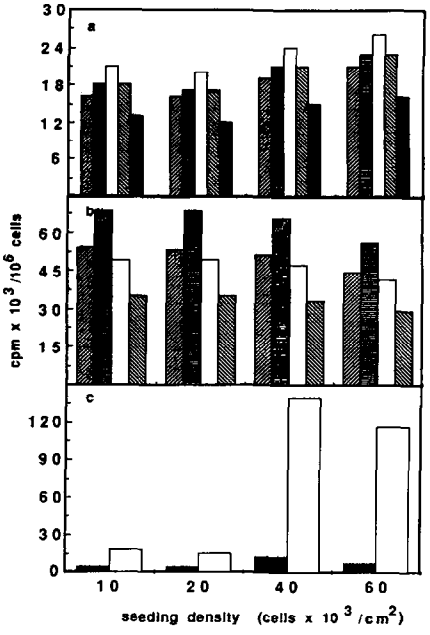

FIG. 4. **Effect of cell density on proteoglycan distribution.** Human bone cells were seeded at 10,000, 20,000, 40,000, and 60,000 cells/cm^2 and steady-state labeled with [^{35}S]sulfate or [^{3}H]GlcN as in Fig. 2 on day 1 after passage. The incorporation of [^{35}S]sulfate into CSPG (▨), HSPG (▩), biglycan (□), decorin (▧), and free glycosaminoglycans (■) was determined as in Fig. 1*b* by liquid scintillation of gel slices for both the cell layer (*a*) and medium (*b*) pools. The incorporation of [^{3}H]GlcN into hyaluronan cell layer (■) and medium (□) was determined as in Fig. 2 (*c*).

after subculture. Both the cell layer (Fig. 4*a*) and medium pools (Fig. 4*b*) show essentially the same PG distribution at the four densities that are equivalent in cell number to days 1, 3, 5, and 9 in long term culture. In contrast, the levels and distribution of hyaluronan changed at the four different densities (Fig. 4*c*). Furthermore, the possibility that the changes in PG steady state labeling with increased time in culture were caused by depletion of medium nutrients (which could, in turn, alter metabolic precursor pools) was investigated by analyzing the PG content of confluent cultures (60,000 cells/cm^2) labeled in the presence of different fetal bovine serum concentrations. When bone cells were labeled with [^{35}S]sulfate for 12 h in medium containing elevated levels of fetal bovine serum (from 10 to 20%), the levels and compartmentalization of the PGs remained relatively constant (data not shown).

Instantaneous Rates of Proteoglycan Synthesis—The instantaneous rate of total PG synthesis was assayed by 30-min pulses on day 2 and day 12 of culture in order to determine if the observed changes were due to an alteration in the metabolic [^{35}S]sulfate pool. Following the addition of fresh medium, triplicate dishes were pulse-labeled for 30 min at 1-h intervals and the incorporation into medium and cell layer fractions determined. The results summarized in Table II show that, on a per cell basis, there was no significant difference between the nanomoles of [^{35}S]sulfate incorporated per h between days 2 and 12.

Pulse Chase Analysis of Proteoglycans—Differences in steady-state labeling could be caused either by increases in the rate of synthesis or by a change in the rate of catabolism of CSPG and biglycan. Bone cells from day 2 of culture were pulse-labeled with [^{35}S]sulfate for 1, 3, and 5 h (Fig. 5) or chased after a 5.5-h labeling for 2, 4, and 6 h (Fig. 6) and the

TABLE II

Instantaneous rates of [^{35}S]sulfate incorporation

Triplicate dishes of human bone cells at either day 2 or 12 after being passaged into mineralization media were pulse-labeled for 30 min by the addition of 100 μCi/ml [^{35}S]sulfate at 0, 1, and 2 h after the addition of fresh medium. The amount of label incorporated was determined by separating polymer-bound [^{35}S]sulfate from free [^{35}S] sulfate on a 25 × 1.0-cm TSK HW 40(S) column as described in text. Cell number was determined in triplicate on unlabeled cohort dishes at the same time.

Day 2			Day 12		
M	CL	Total	M	CL	Total
nmol [^{35}S]sulfate/10^6 cells/h					
0.6	0.4	1.0 ± 0.1	0.2	0.7	0.9 ± 0.1
0.4	0.5	0.9 ± 0.2	0.2	0.7	0.9 ± 0.1
0.7	0.6	1.3 ± 0.2	0.7	0.7	1.4 ± 0.2

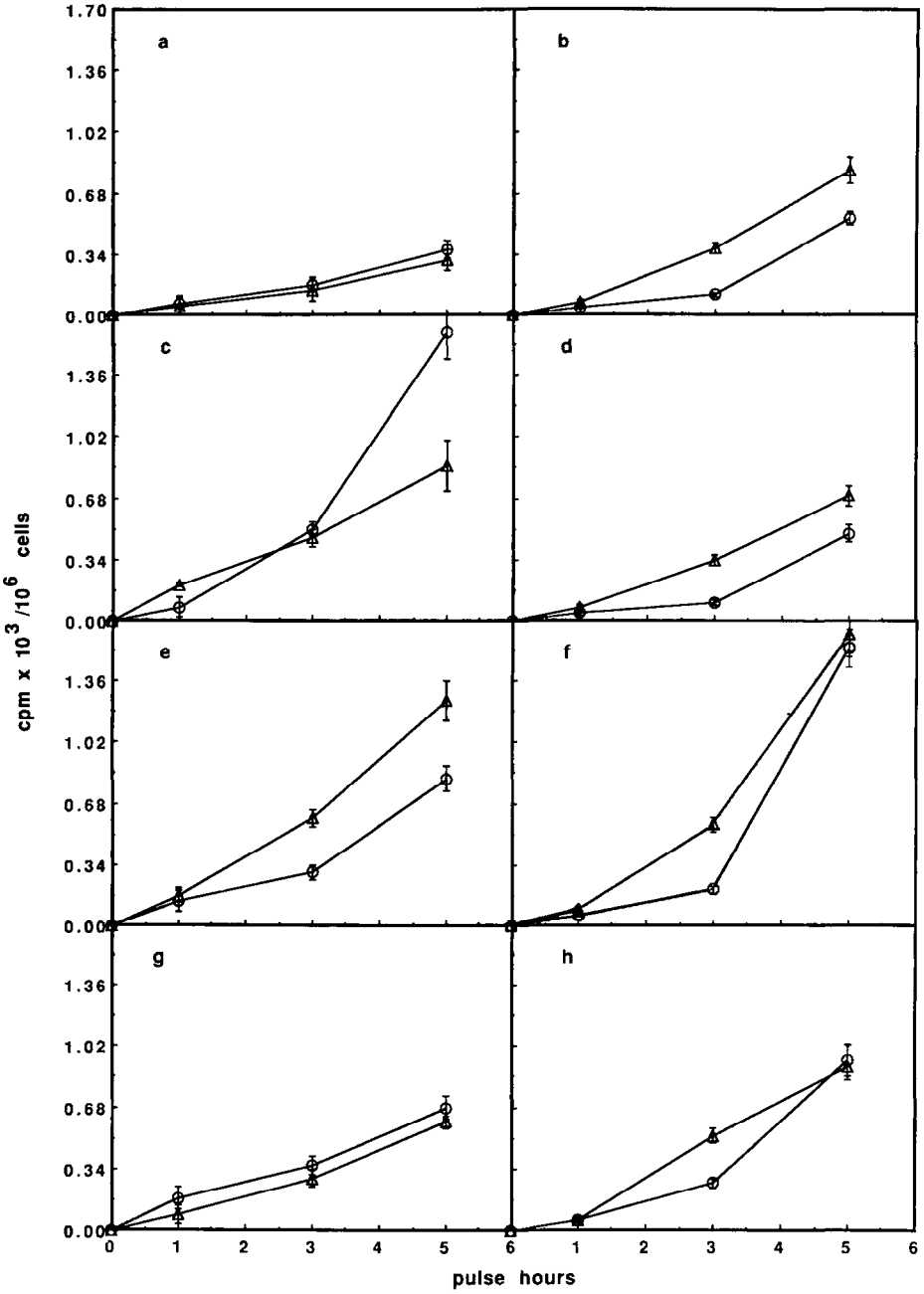

FIG. 5. **Pulse chase comparison of biglycan and decorin.** Human bone cells seeded at 20,000 cells/cm^2 were pulse-labeled on day 2 or 12 for 1, 3, and 5 h. The incorporation of [^{35}S]sulfate into CSPG (*a* and *b*), HSPG (*c* and *d*), biglycan (*e* and *f*), and decorin (*g* and *h*) into the medium (○) and cell layer (△) pools on day 2 (*a, c, e, g*) and day 12 (*b, d, f, h*) was quantitated by liquid scintillation counting of 1-mm SDS-PAGE gel slices as in Fig. 1*b*.

pattern of incorporation into each PG assayed. The four PGs exhibited two distinct biosynthetic patterns with CSPG and decorin possessing one pattern and HSPG and biglycan the other. Both the CSPG and decorin (Fig. 5, *a* and *g*) increased linearly in the cell layer and medium pools during the pulse. Biglycan increased the fastest in the cell layer, accumulating at twice the medium levels during the pulse (Fig. 5*e*), while HSPG in the cell layer reached a plateau by 12 h (data not shown). Both HSPG and biglycan showed an initial lag in their appearance in the medium pools (Fig. 5, *c* and *e*).

The pulse-labeling pattern for each PG was determined after 12 days of culture (Fig. 5, *b, d, f,* and *h*) The general pattern of synthesis for each PG was similar between the 2 days. Again, CSPG and decorin showed equivalent kinetics for pulse (Fig. 5, *b* and *h*) protocols, with both medium and cell layer pools increasing linearly during the pulse experiments. At day 12, a greater amount of all the PGs were retained in the cell layer during both the pulse and chase, consistent with a greater amount of extracellular matrix being formed at that time. The rates of synthesis for HSPG and decorin (Fig. 5, *d* and *h*) were lower while rates for CSPG and biglycan (Fig. 5, *b* and *f*) were only slightly higher by day 12.

When labeling medium was removed and the chase of radiolabeled PGs monitored, the four PGs again fell into two kinetic groups (Fig. 6). The cell layer pools for CSPG and decorin were almost entirely chased into the medium by 6 h of the chase (Fig. 6, *a* and *g*). The sum of medium and cell layer pools for both CSPG and decorin was constant throughout the chase. In contrast, only one-third of biglycan and one-half of HSPG cell layer pools were chased into the medium at 2 days of culture. The two PGs were apparently catabolized as the sum of medium and cell layer pools decreased during the chase (Fig. 6, *c* and *e*). The HSPG was calculated to have a cell layer turnover and catabolism $t_{1/2}$ of 8 h while the apparent $t_{1/2}$ turnover rate of biglycan was 4 h.

Between day 2 and day 12 the apparent rate of turnover of

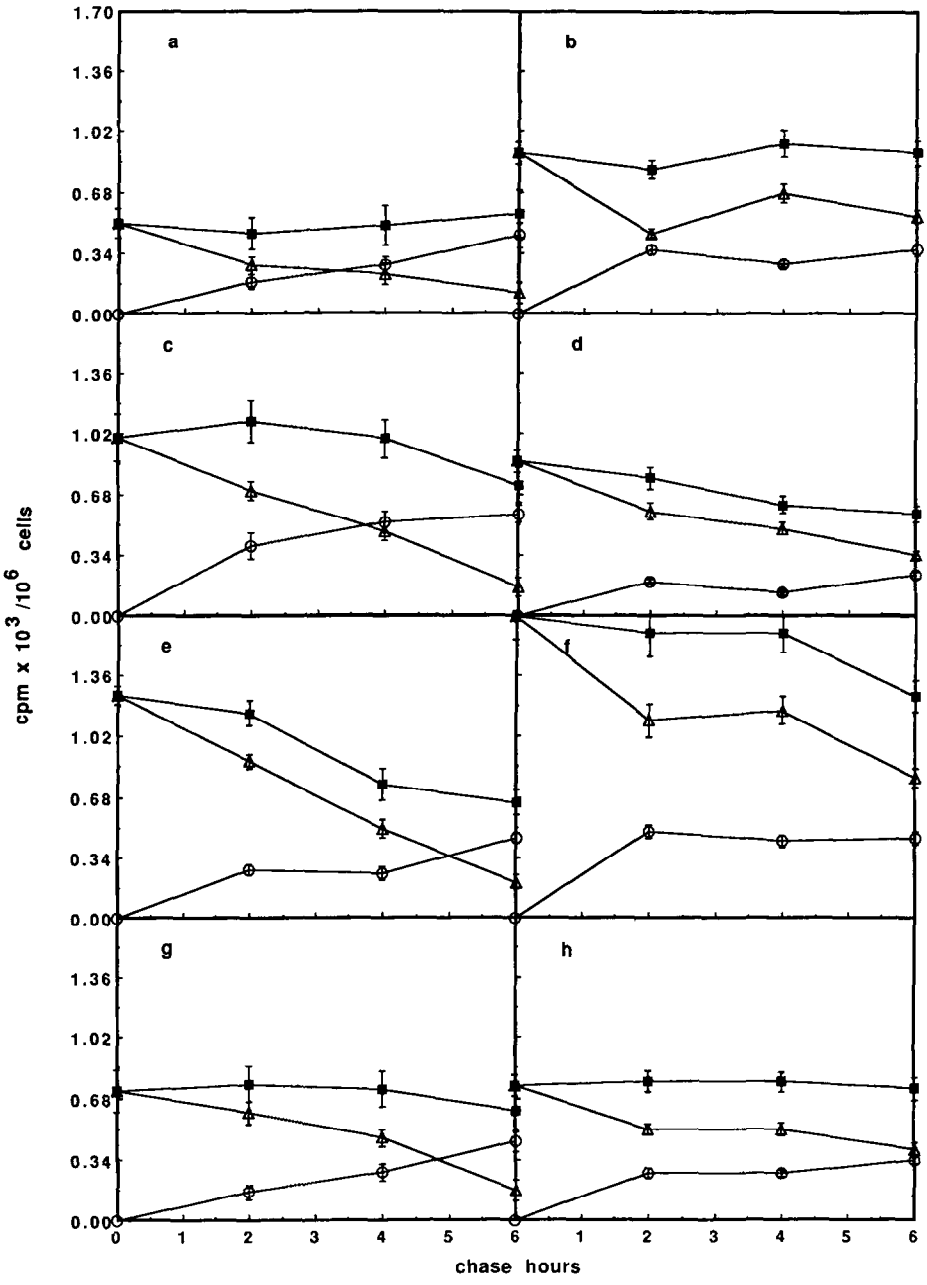

FIG. 6. After 5.5 h pulse labeling, the labeling media was replaced with cold medium and the radiolabeled material was chased for 2, 4, and 6 h. The chase kinetics for CSPG (*a* and *b*), HSPG (*c* and *d*), biglycan (*e* and *f*), and decorin (*g* and *h*) in the medium (○), cell layer (△), and total (medium plus cell layer) pools (■) were determined on day 2 (*a, c, e, g*) and day 12 (*b, d, f, h*) as in Fig. 5.

cell layer biglycan (Fig. 6, *e* and *f*) decreased from 4 to 6 h while HSPG turnover remained the same (Fig. 6, *c* and *d*). Even with the slight increase in synthesis and decrease in catabolism, the increased steady-state labeling pattern of biglycan (Fig. 3*c*) was greater on day 12 then would be calculated based on pulse rates of synthesis and catabolism between the 2 days.

Northern Analysis of Biglycan and Decorin mRNA Levels at Different Times—The above observation that the elevation in proportion of newly synthesized [^{35}S]sulfate-labeled CSPG and biglycan with increasing length of time in culture could not be accounted for entirely by changes in the rate of catabolism alone suggested that regulation of PGs also involved modulation of mRNA and corresponding core protein synthesis. To determine if this changing pattern of PG biosynthesis is reflected in message and core protein levels, the levels of biglycan and decorin mRNA were probed and the incorporation of [^{3}H]leucine/proline into the core proteins were followed. Human cDNA probes encoding biglycan (PG I) and decorin (PG II) (Fisher *et al.*, 1989) were used to screen the relative levels of PG mRNA by Northern analysis as a function of the length of time in culture. The amount of ^{32}P-labeled cDNA probes binding to mRNAs for biglycan, decorin and glyceraldehyde-3-phosphate dehydrogenase was quantitated by cutting the band from the blot and liquid scintillation counting. When the PG messages were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA (a constitutively expressed gene), the levels of decorin mRNA were relatively constant throughout 2 weeks of culture (Fig. 7*a*). The message for biglycan, however, increased rapidly during the first week, reached a plateau during the second week, and fell during the third week of culture (Fig. 7*a*). Comparison of steady-state protein levels as reflected by the sum of medium and cell layer [^{3}H]leucine/proline-PG (Fig. 7*b*), with message levels indicated that both message and intact PG for decorin did not change significantly during 3 weeks in culture. The

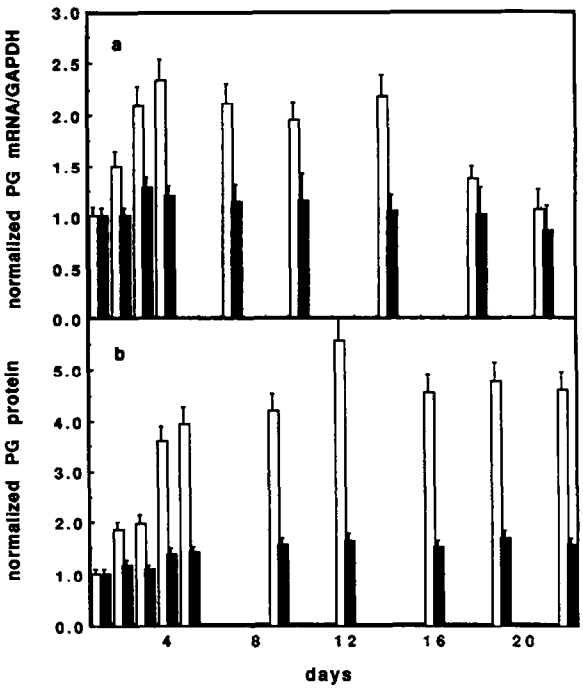

FIG. 7. **Northern analysis of biglycan and decorin mRNA levels with days in culture.** The mRNA from bone cells was extracted on days 1, 2, 3, 4, 7, 9, 14, 18, and 21 of culture. The mRNA was resolved by 1% agarose-formaldehyde gel electrophoresis and then transferred onto nitrocellulose filters, screened with [^{32}P]biglycan, decorin and glyceraldehyde-3-phosphate dehydrogenase probes. The relative levels of biglycan (□) and decorin (■) mRNA were analyzed by liquid scintillation counting of excised bands and normalizing the ^{32}P-radioactivity to that of glyceraldehyde-3-phosphate dehydrogenase (*a*). After separation of [^{3}H]leucine/proline-labeled material using the same column and gradient as in Fig. 1*a*, an aliquot of the proteoglycan pool was analyzed by SDS-PAGE and analyzed by liquid scintillation counting of 1-mm gel slices. The steady-state levels of [^{3}H]leucine/proline-labeled biglycan (□) and decorin (■) were determined on days 1, 2, 3, 4, 5, 9, 12, 16, 19, and 22 of culture (*b*).

mRNA for biglycan, however, rose faster than the corresponding protein and fell in the third week, indicating both transcriptional and translational control of biglycan expression.

DISCUSSION

Like other connective tissue cells, osteoblasts elaborate a collagen-rich extracellular matrix that, in bone, becomes mineralized. We have shown that these cells, in culture, produce PGs that are increasingly cell layer-associated as an extracellular matrix is elaborated with increasing length of time in culture. These bone cells *in vitro* were found to change their pattern of PG metabolism and compartmentalization with time in culture. These results were independent of cell density, media depletion, and labeling pool effects. Changing patterns of extracellular glycosaminoglycans have long been linked with the state of matrix and cell metabolism as well as temporal development (Dingle and Webb, 1965). Of the two small interstitial PGs, biglycan and decorin, the latter (decorin) appears to be produced at constant levels (on a per cell basis) regardless of cell culture time, while the former (biglycan) is rapidly induced within 1–2 days of culture time. The observed temporal changes in biglycan synthesis are preceded by changes in the level of mRNA coding for the PG. There is at present, no evidence to distinguish whether the increase (and subsequent decrease) in biglycan mRNA is from a change in message stability or in alterations of actual levels of transcription. The change in message levels does suggest, however, that the observed alterations in PG metabolism are either genetically programmed or subject to a feedback control system as matrix deposition occurs.

The extracellular space that surrounds a cell may be subdivided into three microenvironments: the cell surface, the pericellular matrix, and the extracellular matrix. The extracellular space *in vitro* is formed through transport of its constituents from the Golgi apparatus to the cell surface, followed by release into the medium. Whether secreted components are actively sequestered or adventitiously trapped to the forming matrix has yet to be resolved. If the matrix was formed by the recapture of soluble secreted proteins into a growing extracellular territorial space, such components would be expected to be secreted at about the same rate (von Wurtemberg and Fries, 1989). Recent evidence supports a model for secretion where proteins are released in bulk phase, whereas organelle-destined proteins are retained by binding to specific ligands in the lumen (Munro and Pelham, 1987; Wieland *et al.*, 1987). The observed coordinate regulation of collagen and alkaline phosphatase metabolism in chick chondrocytes (Habuchi *et al.*, 1985) and the coordinated secretion of both procollagen and fibronectin by fibroblasts (Uchida *et al.*, 1979) is consistent with bulk secretion of proteins destined for the extracellular matrix. The exceptions to this bulk secretion are some proteins destined for secretory granules (Moore *et al.*, 1983) or for one side of polarized cells (Urban *et al.*, 1987). In these instances, the proteins were actively sorted.

The transport of certain PGs to the cell surface may be analogous to protein transport where two routes are possible: bulk secretion and sequestering of ligand-associated products that are destined for organelles. The plasma membrane is, after all, a cellular organelle, and PGs possessing vectorial secretion have been described in rat uterine epithelial cells (Carson *et al.*, 1988). The plasma membrane association of HSPGs through phosphatidylinositol (Ishihara *et al.*, 1987; Carey and Evans, 1989) and the localization of CSPGs to the plasma membrane (Hedman *et al.*, 1983; David *et al.*, 1989) suggest that certain PGs are specifically targeted for the plasma membrane space. The identification of membrane receptors for extracellular matrix dermatan sulfate PGs (Glossl *et al.*, 1983; Schmidt and Buddecke, 1985) and hyaluronan (Alho and Underhill, 1989) may provide the cell surface with a mechanism for recapturing secreted PGs. The present observations of a bone cell CSPG and decorin that have pulse-chase kinetics (*a*) where the medium and cell layer pools linearly increase in parallel and (*b*) where the cell layer pool can be chased almost entirely into the medium at an early time in matrix creation suggest that these two PGs are behaving as secretory PGs and participating in events occurring in the extracellular matrix. Once an extracellular matrix is well elaborated, these two PGs are retained to a greater extent in the cell layer during the chase, kinetics again consistent with the localization of these PGs to the extracellular matrix. It is interesting to note that the interaction of the smaller dermatan sulfate PG (decorin, PG II) with collagen in the extracellular matrix has been described previously (Scott, 1988; Vogel *et al.*, 1987), data consistent with our observed biosynthetic kinetics. Both HSPG and biglycan, on the other hand, lag in appearance as medium [^{35}S]PGs during short term labeling. Such a transient delay in secretion has been suggested to reflect necessary processing reactions prior to HSPG secretion by rat hepatocytes (Bienkowski and Conrad, 1984; Ishihara *et al.*, 1986) and granulosa cells (Yanagashita and Hascall, 1987). The metabolic properties observed for

HSPG in this study (that of the cell layer pool reaching a steady-state during pulse labeling and rapid turnover of the cell layer pool during the chase) suggest that the bone HSPG, like the rat hepatocyte and ovarian granulosa cell HSPG, is synthesized and transported to the cell surface where a portion of the pericellular pool can either escape (or be released) into the medium or undergo endocytosis and catabolism. The similar compartmentalization and turnover of bone biglycan suggest that it is also a pericellular PG whose levels are regulated by both synthesis and catabolism. The current data suggest that CSPG and decorin release into the medium and extracellular matrix association *in vitro* reflect a bulk secretion process, while HSPG and biglycan are sequestered to the pericellular matrix (perhaps via ligand association).

In the present study, sparsely seeded human bone cells by day 2 of subculture are in the process of rapidly synthesizing components for the nascent matrix. It is during subsequent days of culture that a dense extracellular matrix is elaborated and the cells reach confluence (day 9) and begin to overlay each other. Once cells become multilayered, the rate of proliferation decreases while the levels of extracellular (CSPG and decorin) and pericellular matrix (HSPG and biglycan) PGs either remain constant or increase (in the case of biglycan). The finding that hyaluronan synthesis is uncoupled from that of PGs and actually decreases with length of time in culture indicates that hyaluronan may be involved in an early event of matrix formation. It may be that, *in vitro,* hyaluronan functions to capture extracellular space for subsequent matrix deposition. Thus the density dependence of hyaluronan steady state levels might be indicative of the requirement for more space to be reserved ("captured") for matrix formation when cells are seeded at high densities. The lack of a cell density effect on PG levels and compartmentalization measured shortly after replating at different densities may be explained by the absence of a deposited matrix which would 1) adsorb PGs (such as CSPG and decorin) and 2) influence the metabolism of the apparently pericellular PGs (HSPG and biglycan). The decrease in hyaluronan and increase in PG steady state levels that occurs with length of time in culture would then reflect the deposition of an extracellular matrix in the captured space. The *in vitro* mineralization of adult human bone cell-derived extracellular matrix requires long term culture, on the order of 2 months[3]; thus the current studies are concerned with correlating hyaluronan and PG metabolism with matrix formation rather than mineralization. However, having defined the patterns of hyaluronan and PG metabolism and compartmentalization in this *in vitro* system, the interpretation of the effect of different developmental ages and diseased states of mineralization on human bone cell metabolism and matrix formation *in vitro* will be greatly facilitated.

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